

Peptide YY Receptors in the Proximal Tubule PKSV-PCT Cell Line Derived from Transgenic Mice

RELATION WITH CELL GROWTH*

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Thierry Voisin[‡], Marcelle Bens[‡], Françoise Cluzeaud[‡], Alain Vandewalle[‡], and Marc Laburthe[‡]

From the [‡]Unité de Biologie et Physiologie des Cellules Digestives, Institut National de la Santé et de la Recherche Médicale, Unité 239 and the [¶]Unité de Physiologie et Pathologie Cellulaire du Néphron, Institut National de la Santé et de la Recherche Médicale, Unité 246, 75018 Paris, France

Receptors for peptide YY (PYY) were identified in the PKSV-PCT renal proximal tubule cell line, derived from transgenic mice (SV40 large T antigen under the control of the rat L-type pyruvate kinase 5'-regulatory sequence). Binding of [¹²⁵I-Tyr³⁶]monoiodo-PYY ([¹²⁵I]PYY) to cell was specific, saturable, and reversible. The order of potency for peptides for inhibiting [¹²⁵I]PYY binding was: PYY > neuropeptide Y (NPY) = PYY(13-36) >> pancreatic polypeptide. A single class of receptors was observed with a K_d of 0.37 ± 0.05 nM and a B_{max} of 103 ± 10 fmol/mg protein. After cross-linking, electrophoresis of covalent [¹²⁵I]PYY-receptor complexes revealed a single band of M_r 50,000. PYY receptors were exclusively present at the basolateral membrane surface of polarized cells and were coupled negatively to adenylcyclase by a pertussis toxin-sensitive G protein. PKSV-PCT cell growth and T antigen expression could be modulated by D-glucose in the medium. PYY receptors were exclusively expressed in proliferative cells cultured in the presence of D-glucose. PYY receptors disappeared in the absence of D-glucose and were expressed again when proliferation was activated by reintroduction of D-glucose. PYY stimulated cell growth (17–26% increase) and promoted [methyl-³H]thymidine incorporation into DNA (64% increase; $ED_{50} = 5$ nM PYY) of cells grown in D-glucose-enriched medium. This latter effect of PYY was largely reversed by pretreatment of cells with pertussis toxin. These findings suggest that PYY receptors play a role in epithelial cell growth.

Receptors for peptide YY (PYY),¹ a 36-amino acid polypep-

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§ To whom correspondence should be addressed: INSERM U239, Faculté de Médecine Xavier Bichat, 75018 Paris, France. Tel.: 33-1-44-85-61-32; Fax: 33-1-42-28-87-65.

¹ The abbreviations used are: PYY, peptide YY; [¹²⁵I]PYY, [¹²⁵I-Tyr³⁶]monoiodo-PYY; NPY, neuropeptide Y; PP, pancreatic polypeptide; Tag, large T antigen; L-PK, L-type pyruvate kinase; GTP_γS, guanosine 5'-O-(thiotriphosphate); G_i, inhibitory regulatory GTP-binding protein of adenylcyclase; G_s, stimulatory regulatory GTP-binding protein of adenylcyclase; BSA, bovine serum albumin; TLCK, N α -p-tosyl-L-lysine chloromethyl ketone; DSS, disuccinimidyl suberate.

tide, were first described by Laburthe *et al.* (1) in the rat small intestine. Compared to the other receptors for the PP-fold family of peptides, this receptor has been defined as PYY-prefering because it displays a 5–10-fold higher affinity for PYY than for NPY (1, 2). It is different from the three subtypes of NPY receptors identified in various tissues (3–5) but resembles the Y2-subtype (3, 4) in that it binds long COOH-terminal fragments of PYY and NPY (4). The intestinal PYY receptor has been further characterized as a M_r 44,000 glycoprotein (6).

In small intestinal epithelium from the rat species, PYY or NPY binding to PYY receptors results in a potent inhibition of cAMP production (7, 8) and of fluid and electrolyte secretion (2). These observations led us to propose that intestinal PYY receptors may have pharmacological value for the development of new antidiarrheal drugs (2). This prompted us to investigate the cellular site of action of PYY in small intestinal epithelium. In keeping with the antisecretory effect of PYY, we evidenced abundant PYY receptors in crypt cells where intestinal Cl[−] secretion is believed to take place (8). We also pointed out an intriguing observation: PYY receptors were almost exclusively present in proliferative crypt cells and appeared to be quenched when epithelial cells migrated onto the villi and stop to divide (8). Unfortunately, the relationship between PYY receptors and epithelial cell proliferation could not be further documented because of the absence of intestinal epithelial cell line expressing PYY receptors (2). This is probably related to the proximo-distal gradient of PYY receptors in intestine (1). Indeed, PYY receptors are expressed mainly in the upper small intestine and not in colon (1, 2) whereas most established cell lines, such as Caco-2 or HT-29 cells, displaying typical features of intestinal epithelial cell differentiation have been derived from human colonic carcinomas (9, 10).

While we looked for an epithelial cell line in culture expressing PYY receptors, we considered renal tubule epithelial cells since NPY has been shown to affect functions of the kidney (11–14), and PYY receptors have been evidenced in isolated rabbit kidney proximal tubular cells (15).

We report here the characterization of PYY-prefering receptors in an established renal cell line, the PKSV-PCT cells, derived from microdissected proximal convoluted tubules of kidneys from the L-PK/Tag1 transgenic mice harboring the SV40 large T antigen placed under the control of the rat L-type pyruvate kinase 5'-regulatory sequence (16). Previous studies indicated that expression of T antigen (Tag) and thereby cell growth are dependent upon the concentration of D-glucose in the culture medium without alteration of tissue-specific functions (17, 18), and that cells remained fully

differentiated under the conditions of activation or inactivation of Tag transcripts (17). This feature made PKSV-PCT cells a suitable cellular model for further analyzing the putative relationship between epithelial cell growth and PYY receptors. In this study, we provide evidence for the expression of a *M*, 46,000 PYY receptor negatively coupled to adenylyl cyclase by a pertussis toxin-sensitive *G*_i protein. This receptor is exclusively localized on the basolateral surface membrane and is only expressed when cells proliferate upon transgene activation. Conversely, PYY stimulates cell growth in PKSV-PCT cells cultivated in D-glucose-enriched culture medium.

EXPERIMENTAL PROCEDURES

Materials—Synthetic porcine PYY, porcine NPY, rat PP, and porcine PYY(13-36) purchased from Peninsula Laboratories, Belmont, CA, were used in all experiments. The 1-34 synthetic fragment from bovine parathormone was from Calbiochem, France. Na[¹²⁵I] (IMS300) was from Amersham Corp. and [methyl-³H]thymidine from Du Pont-New England Nuclear. The cAMP radioimmunoassay kit (no. 7938) was purchased from the Institut Pasteur (Lyon, France). The culture media DMEM and Ham's F-12 were purchased from Life Technologies, Inc. The culture reagents, transferrin, sodium selenate, dexamethasone, triiodothyronine, insulin, glutamine, forskolin, epidermal growth factor, HEPES, phenylmethylsulfonyl fluoride, TLCK, bacitracin, GTP, GDP, GTP-γS, ATP, pertussis toxin, and the other highly purified chemicals used were purchased from Sigma. BSA (Pentex, Fraction V) was obtained from Miles Laboratories (Elkart, NJ) and DSS from Pierce Chemical Co. Anti-α₁₁/α₁₂ (AS7), anti-α₃/α₆ (EC2), anti-α₄ (A572), and anti-β (U49) antibodies were from Du Pont-New England Nuclear.

Cultured Cells—The PKSV-PCT cell line was derived from microdissected proximal convoluted tubules from kidney of transgenic mouse (L-PK/Tag1) carrying the large T and small t antigens of the simian virus 40 (SV40) placed under the control of the rat L-type pyruvate kinase promoter gene (16). PKSV-PCT cells were currently cultured in a modified culture medium (DMEM:Ham's F-12, 1:1 (v/v); 30 nM sodium selenate; 5 μg/ml transferrin; 2 mM glutamine; 50 nM dexamethasone; 1 nM triiodothyronine; 10 nM epidermal growth factor; 2% fetal calf serum; 20 mM HEPES, pH 7.4), supplemented with 5 μg/ml insulin and 20 mM D-glucose at 37 °C in 5% CO₂, 95% air atmosphere as described (18). Previous studies have shown that such a condition of culture with D-glucose-enriched medium favors the activation of Tag transcripts and cell growth. In some series of experiments, sets of subconfluent PKSV-PCT cells grown in D-glucose-enriched medium were further cultivated in this medium, in which D-glucose and fetal calf serum were replaced by 2% dialyzed fetal calf serum, 2 mM oxaloacetate, and 5 mM pyruvate (D-glucose-deprived medium) (17).

All studies were performed between the 35th and 60th passages on sets of cells seeded on plastic Petri dishes (60- or 100-mm diameter) or on semipermeable transparent filters precoated with collagen (Transwell-COL, 0.4-μm pore size, 1.2-cm² diameter; Costar Europe Ltd., Badhoevedorp, The Netherlands).

Preparation and Purification of Iodinated PYY—Synthetic PYY was radiolabeled with Na[¹²⁵I] using the chloramine T method, as described elsewhere (19). The purification of radiolabeled PYY was performed using reverse phase high performance liquid chromatography as previously described (19). Two principal tracers were obtained in these conditions: the [¹²⁵I-Tyr¹]monoiodo-PYY and the [¹²⁵I-Tyr³⁶]monoiodo-PYY. Fractions containing the radiolabeled tracer were stored at -20 °C in a 0.1 M acetic acid buffer with 0.1% BSA. The [¹²⁵I-Tyr³⁶]monoiodo-PYY (referred to as [¹²⁵I]PYY below) showed a much higher level of specific binding to PKSV-PCT cell membranes than [¹²⁵I-Tyr¹]monoiodo-PYY and was therefore used for all binding experimental procedures as previously described (19). [¹²⁵I]PYY was used during the 1st month following labeling without a significant loss of binding activity.

Preparation of Particulate Fraction of PKSV-PCT Cells—A particulate fraction was prepared from PKSV-PCT cells seeded in 100-mm plastic Petri dishes grown in D-glucose-enriched medium for 6 days or grown in this medium for 4 days and then further cultivated in D-glucose-deprived medium for 48 h. In all cases, cells were washed three times with 0.13 M phosphate-buffered saline (pH 7.0), then harvested using a rubber policeman and centrifuged at 2,000 × *g* for 5 min at 4 °C. The cell pellet was then exposed for 30 min to

hypotonic 5 mM HEPES buffer (pH 7.4). Thereafter, aliquots of cell suspension were centrifuged at 20,000 × *g* for 15 min, washed with 20 mM HEPES buffer (pH 7.4), pelleted, and stored at -80 °C until used. This particulate fraction from cell homogenate will be referred to as membrane preparation.

PKSV-PCT Cell Treatment with Pertussis Toxin—In some sets of experiments, confluent cells grown in 100-mm diameter plastic Petri dishes were treated overnight (16 h) with pertussis toxin (0.4 μg/ml) which was added to the culture medium. A particulate fraction was then prepared as described above and used immediately for binding experiments. A similar procedure was applied to sets of confluent cells grown in 12-well trays before cellular cAMP assay.

Binding of [¹²⁵I]PYY to Cells Grown on Filters—PKSV-PCT cells seeded on semipermeable filters coated with collagen were grown until confluence in D-glucose-enriched culture medium. Binding of [¹²⁵I]PYY was then conducted either on the apical or the basolateral side of confluent cell monolayers grown on filters. Briefly, the apical side (inside of the chamber) or the basal side (outside of the chamber) of the cell monolayer were incubated with 0.5 and 1.5 ml of culture medium, respectively, containing 0.1% (w/v) BSA and 0.1 nM [¹²⁵I]PYY for 60 min at 37 °C. The nonspecific binding was determined by addition of 1 μM unlabeled PYY. At the end of the incubation, the inside and outside of the chamber were washed five times with 1 and 1.5 ml of ice-cold phosphate-buffered saline, respectively. After drying, the filters were detached and then counted with a γ counter. The specific binding was calculated as the difference between the amount of [¹²⁵I]PYY bound in the absence (total binding) or presence (nonspecific binding) of an excess (1 μM) of unlabeled PYY. Values are expressed as counts/min/well.

Binding of [¹²⁵I]PYY to Membrane-bound Receptors—Binding of [¹²⁵I]PYY to membranes was conducted as previously described (1, 7, 8). Briefly, membranes (200 μg of protein/ml) were incubated for 90 min at 30 °C in 250 μl of incubation buffer (20 mM HEPES buffer, pH 7.4, 2% (w/v) BSA, 0.1% (w/v) bacitracin, 17 mg/liter phenylmethylsulfonyl fluoride, 10 mg/liter TLCK, 10 mg/liter pepstatin, 10 mg/liter leupeptin, and 100 mg/liter bacitracin) containing 0.05 nM [¹²⁵I]PYY with or without unlabeled PYY or other competing peptides. Preliminary kinetic studies have shown that association of [¹²⁵I]PYY to PKSV-PCT membrane receptors reached a plateau after 60 min of incubation which was maintained for at least 180 min. At the end of the incubation (60 min), aliquots (150 μl) of membranes were mixed with 150 μl of ice-cold incubation buffer. Bound and free peptides were separated by centrifugation at 20,000 × *g* for 10 min, and membrane pellets were washed twice with 10% (w/v) sucrose in 20 mM HEPES. The radioactivity was then counted with γ counter. The nonspecific binding represented about 2% of total radioactivity. All binding data were analyzed using the LIGAND computer program developed by Munson and Rodbard (20).

Cross-linking of Bound [¹²⁵I]PYY to Membrane Receptors and SDS-Polyacrylamide Gel Electrophoresis—PKSV-PCT cell membranes containing bound [¹²⁵I]PYY were suspended in 1 ml of 20 mM HEPES buffer (pH 7.4) and incubated with 1 mM DSS for 15 min at 4 °C as described elsewhere (6). The cross-linked material was then treated as previously described (6) before SDS-polyacrylamide gel electrophoresis which was run according to the procedure of Laemmli (21). All cross-linked extracts containing up to 100 μg of protein were loaded onto a 12% polyacrylamide gel with a 5% stacking gel as described in detail elsewhere (21). Gels were stained, dried, and exposed for 7–15 days at -80 °C to a Trimax type XM film (3M) with a 3M Trimax intensifying screen. Gels were calibrated with the following molecular weight marker proteins from Bio-Rad: myosin (200,000), phosphorylase *b* (97,000), BSA (68,000), ovalbumin (43,000), carbonic anhydrase (29,000), lactoglobulin (18,400), and lysozyme (14,300).

Adenylyl cyclase Assay and cAMP Measurement—Adenylyl cyclase was assayed as described elsewhere (9). Membrane preparations from PKSV-PCT cells (10–30 μg of protein/ml) grown in different conditions, as described in the text and legend of figures, were incubated in a volume of 250 μl of 20 mM HEPES buffer (pH 7.4) containing 2% BSA, 0.1% bacitracin, 1 mM ATP, 5 mM MgCl₂, 0.2 mM 3-isobutyl-1-methylxanthine, 10 μM GTP, and 100 mM NaCl. Reactions were started with addition of membranes. Incubations were performed at 30 °C for 15 min. Reactions were stopped and cyclic AMP (cAMP) was measured by radioimmunoassay as described elsewhere (8).

Cellular cAMP content was assayed as previously described (22). Cells in 12-well trays were incubated in 1 ml of DMEM without or with 0.1 μM PYY and/or 10 μM forskolin for 7 min at 37 °C. The medium was rapidly removed and 1 ml of ice-cold 95% ethanol/5%

formic acid was added. Supernatants were evaporated to dryness and cAMP was determined by radioimmunoassay Pasteur kit. Data are reported as picomol of cAMP/mg of protein.

Immunoblotting of α - and β -Subunits of G Proteins—Membranes (50 μ g of protein) were solubilized as described elsewhere (23). Then, samples were alkylated prior to electrophoresis for enhancing the clarity and hence resolution of polypeptide bands (24). Samples were heated at 100 °C for 5 min and proteins were separated in a 10% polyacrylamide gel as described elsewhere (23). Proteins were transferred to nitrocellulose as previously described (25). The nitrocellulose sheets were washed and incubated with 125 I-labeled goat antibodies to rabbit IgG in 50 mM Tris-HCl, 500 mM NaCl, and 0.02% NaN₃ for 2 h. After extensive washing, nitrocellulose sheets were dried before autoradiography (23).

Cell Growth and [methyl- 3 H]Thymidine Incorporation—Cell growth kinetics were estimated by cell count and [methyl- 3 H]thymidine (specific activity, 25 Ci/mmol) incorporation. PKSV-PCT cells, seeded at the same concentration (2×10^4 cells/plate) in 35-mm individual plastic Petri dishes and counted daily, were detached with 0.05% trypsin, 0.02% EDTA, resuspended, and counted in a hemocytometer. The influence of various culture conditions (D-glucose-enriched or D-glucose-deprived medium and 24 h of incubation with PYY) on DNA synthesis was estimated by measuring the [methyl- 3 H]thymidine incorporated into trichloroacetic acid-insoluble cell fractions. Cell monolayers from 12-well trays, after treatment as described, were incubated for 6 h with [methyl- 3 H]thymidine (0.5 μ Ci/well), rinsed three times with 1 ml of ice-cold phosphate-buffered saline, and incubated with 1 ml 5% trichloroacetic acid for 30 min at 4 °C. Trichloroacetic acid was discarded and cells were further incubated for 30 min at 37 °C with 1 ml of 0.3 N NaOH before neutralization with acetic acid, and tritium was measured by scintillation counting. All experiments were performed in triplicate, and results are expressed as counts/min/ 10^6 cells.

Electron Microscopy—PKSV-PCT cells cultured on Costar filters coated with collagen in D-glucose-enriched medium were processed for electron microscopy as described elsewhere (18).

Protein Determination—Proteins were measured using a protein assay kit (Bio-Rad) based on the method of Bradford (26) with BSA as a standard.

Statistical Analysis—Results are expressed as means \pm S.E. from (*n*) separate experiments. Statistical significance between groups was calculated by the Student's *t* test.

RESULTS

PYY Receptors in PKSV-PCT Cells

Specificity, Stoichiometry, Expression at the Basolateral Membrane, and Molecular Characterization—In the condition of culture where PKSV-PCT cells were cultivated in the presence of D-glucose-enriched culture medium, PYY receptors could be evidenced in PKSV-PCT cell membrane preparations. These receptors discriminate between PYY, NPY, PP, and the PYY fragment PYY(13-36) (Fig. 1A). The peptide concentrations that induced half-maximal inhibition of [125 I]PYY binding (IC_{50}) are as follows: PYY ($IC_{50} = 0.3 \pm 0.1$ nM) < NPY ($IC_{50} = 9.0 \pm 0.7$ nM) = PYY(13-36) ($IC_{50} = 10.0 \pm 1.0$ nM) << PP ($IC_{50} > 1 \mu$ M). These results clearly define the presence of a PYY-preferring receptor in PKSV-PCT cells exhibiting similar specificity as the one previously identified in rat small intestinal epithelium (1, 2, 7, 8).

[125 I]PYY binding to PKSV-PCT cell receptors was saturable. This was shown by studying the concentration dependence of PYY binding at equilibrium (Fig. 1B). Scatchard analysis gave a straight line supporting the conclusion that PYY bound to a single population of sites in PKSV-PCT cell membranes. The binding parameters obtained from Scatchard plots were the following: $K_d = 0.37 \pm 0.05$ nM; $B_{max} = 103 \pm 10$ fmol/mg of protein.

Binding of [125 I]PYY was also conducted on apical and basolateral sides of polarized PKSV-PCT cells grown on permeable filters in the presence of D-glucose-enriched medium. As shown in Fig. 2 (upper panel) confluent PKSV-PCT

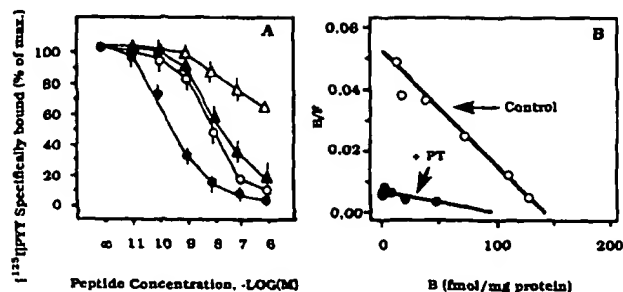


FIG. 1. Peptide specificity of PYY receptors on PKSV-PCT cell membranes and Scatchard analysis of PYY binding. A, peptide specificity of PYY receptors was investigated on membranes from PKSV-PCT cells grown in D-glucose-supplemented medium. Membranes were incubated with 0.05 nM [125 I]PYY and increasing concentrations of unlabeled PYY (●), NPY (○), PP (△) or PYY(13-36) (▲). Nonspecific binding was determined in the presence of 1 μ M unlabeled PYY. Results are the means \pm S.E. from three experiments. B, saturation analysis was conducted as described under "Experimental Procedures" in the presence of a fixed concentration of [125 I]PYY (0.05 nM) and increasing concentrations of unlabeled PYY. Binding experiments were performed on membranes from control cells (○) or cells pretreated overnight with 0.4 μ g/ml pertussis toxin (●). Scatchard plots were analyzed using the LIGAND computer program (20). Results shown are from a typical experiment. Two other experiments gave similar results.

cells form a monolayer of highly polarized epithelial cells separated by tight junctions and presenting numerous microvilli located at the apical membrane surface (17). When [125 I]PYY was added in the medium bathing the apical surface, no specific tracer binding was observed (Fig. 2, lower panel). In sharp contrast, a high amount of specifically bound tracer was measured when [125 I]PYY was added in the medium bathing the basolateral surface (Fig. 2, lower panel). Wherever the tracer was added (apical or basolateral side of the cells), no significant amount of radioactivity was recovered on the opposite side suggesting that cells were impermeable for the radioactive ligand (data not shown).

Further experiments were conducted to identify the PYY receptor at the molecular level on cell membrane preparations from PKSV-PCT cells grown in the presence of D-glucose-enriched medium. For that purpose, the cross-linker disuccinimidyl suberate (DSS) was used to covalently attach [125 I]PYY to receptor sites. The subsequent SDS-polyacrylamide gel electrophoresis analysis of membrane proteins revealed a single band of *M*_r 50,000 (Fig. 3). The labeling of this band is specific in that it was abolished by 1 μ M unlabeled PYY. Assuming one molecule of [125 I]PYY (*M*_r 4,000) was bound per molecule of receptor, the calculated intrinsic molecular weight of the receptor was about 46,000.

Coupling to a Pertussis Toxin-sensitive G Protein and Inhibition of cAMP Production—Several lines of evidence indicate that PYY receptors in PKSV-PCT cells are coupled to a pertussis toxin-sensitive G_i protein resulting in inhibition of adenylylcyclase and of subsequent production of cellular cAMP: (i) after overnight treatment of PKSV-PCT cells grown in D-glucose-enriched medium to which was added pertussis toxin (0.4 μ g/ml), Scatchard analysis of PYY binding to cell membranes revealed a 7-fold decrease in receptor affinity ($p < 0.003$) and a 1.4-fold decrease in receptor capacity (nonsignificant) as compared to control values (Fig. 1B). Similar effect of pertussis toxin on peptide binding to receptors was previously observed for galanin binding to the insulin-secreting cell line Rin m 5F (27, 28). It may be suggested that association of receptors with G_i proteins is necessary for high affinity binding of peptides to receptors. When pertussis

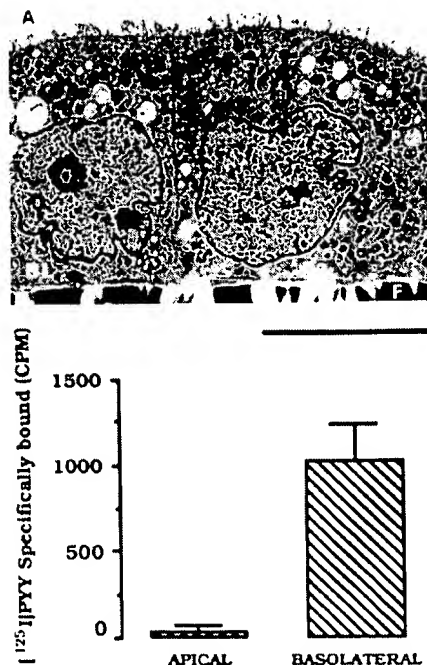


FIG. 2. Binding of $[^{125}\text{I}]\text{PYY}$ to basolateral and apical membranes of polarized PKSV-PCT cells grown on permeable filters. Upper panel, electron microscopic appearance of PKSV-PCT cells grown on semipermeable filter. The cells grown on porous filter, formed regular monolayers of cuboid cells with apical membranes bearing numerous microvilli. The cells are separated by closely apposed lateral membranes with tight junctions and desmosomes (18). A represents the apical side and BL represents the basolateral side of polarized PKSV-PCT cells grown on permeable filter. F. Bar = 10 μm . Lower panel, confluent PKSV-PCT cells grown in D-glucose-enriched medium on permeable filters were incubated with 0.1 nM $[^{125}\text{I}]\text{PYY}$ applied to the apical or basolateral side of the cells for 60 min at 37 $^{\circ}\text{C}$. The nonspecific binding was determined in the presence of 1 μM unlabeled PYY in the incubation medium as described under "Experimental Procedures." Results are expressed as counts/min of tracer specifically bound. Values are the means \pm S.E. from five experiments.

toxin disrupts this association, the receptor converts into a low affinity state for the peptide; (ii) at maximally active dose (10^{-3} M), guanine nucleotides inhibited by 65% the binding of $[^{125}\text{I}]\text{PYY}$ to receptors in membranes prepared from PKSV-PCT cells grown in D-glucose-enriched culture medium. The following order of potency was observed $\text{GTP}\gamma\text{S}$ ($\text{IC}_{50} = 0.03$ μM) $>$ GTP ($\text{IC}_{50} = 0.5$ μM) = GDP ($\text{IC}_{50} = 0.5$ μM). In contrast, ATP failed to alter PYY binding. GTP increased the dissociation rate of $[^{125}\text{I}]\text{PYY}$ -receptor complexes by decreasing the first order dissociation constant k_{-1} from 0.011 min^{-1} (control without GTP) to 0.005 min^{-1} in the presence of 0.1 mM GTP (data not shown); (iii) In the condition of culture when PKSV-PCT cells were cultivated in the presence of D-glucose-enriched culture medium, PYY (0.1 μM) reduced forskolin-stimulated cAMP production by 45% (Fig. 4A and Table I), and overnight treatment of PKSV-PCT cells with pertussis toxin (0.4 $\mu\text{g}/\text{ml}$) blunted the PYY inhibitory effect on forskolin-stimulated cAMP production; (iv) PYY (1 μM) inhibited by about 50% the forskolin-stimulated adenylylcyclase activity in membranes prepared from PKSV-PCT cells cultured in D-glucose-enriched medium (Fig. 4B). This inhibitory effect was completely reversed when cells had been previously incubated overnight with pertussis toxin (data not shown). Similar inhibitory effect of PYY (data not shown) was achieved on adenylylcyclase activity stimulated by para-

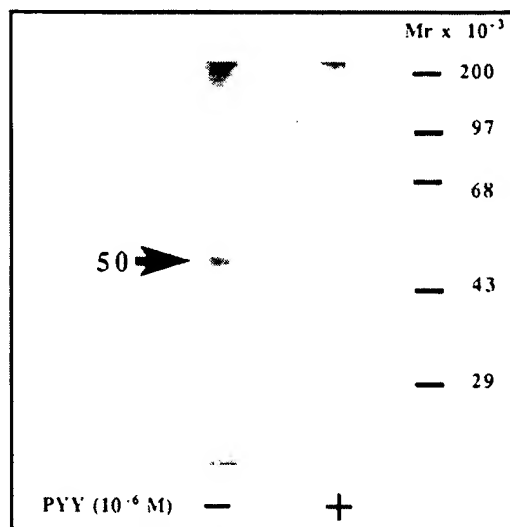


FIG. 3. Covalent labeling of PKSV-PCT cell membranes by $[^{125}\text{I}]\text{PYY}$. Membranes prepared from PKSV-PCT cells grown in D-glucose-enriched medium were incubated with $[^{125}\text{I}]\text{PYY}$ in absence or presence of 1 μM unlabeled PYY. After washing, membranes were treated with 1 mM DSS and submitted to SDS-polyacrylamide gel electrophoresis as described under "Experimental Procedures." Gels were calibrated with the following molecular weight marker proteins: myosin (200,000), phosphorylase b (97,000), BSA (68,000), ovalbumin (43,000), and carbonic anhydrase (29,000).

thormone (10^{-7} M), a hormone specifically acting on PKSV-PCT cells (17); (v) finally the three subtypes of G_i proteins are present in membranes from PKSV-PCT cells since Western blotting revealed α_{i1} , α_{i2} , and α_{i3} subunits (Fig. 5).

Expression of PYY Receptors as a Function of Transgene Activation and Cell Proliferation

The established PKSV-PCT cells derived from transgenic mice harboring Tag under the control of the 5'-regulatory sequence of the L-PK gene maintained *in vitro* the ability of transgene activation by D-glucose. It could be demonstrated in these cells that the expression of Tag transcripts as well as cell growth were dependent upon the concentration of D-glucose present in the culture medium without altering the proximal tubule cell-specific functions (17, 18).

Fig. 6 summarizes the cell growth kinetics and levels of $[\text{methyl-}^3\text{H}]\text{thymidine}$ incorporation in PKSV-PCT cells grown with or without D-glucose in the culture medium. In the presence of D-glucose, PKSV-PCT cells exhibited a high rate of cell doubling, whereas replacement of D-glucose by neoglucogenic substrates (pyruvate and oxaloacetate) at different stages of cell growth resulted in an arrest of cell proliferation. As already observed (17, 18), this phenomenon could be reversed since reactivation of cell growth occurred when D-glucose was reintroduced in the culture medium (Fig. 6A).

In accordance with these observations, the level of $[\text{methyl-}^3\text{H}]\text{thymidine}$ incorporation into trichloroacetic acid-insoluble material decreased by 70% when subconfluent cells are cultured in the absence of D-glucose when compared to the same set of cells grown in D-glucose-enriched medium (Fig. 6B). Moreover, transfer of the cells from D-glucose-deprived to D-glucose-enriched medium restored the levels to that observed on cells first grown in the D-glucose-enriched medium. Arrest of cell growth and the observed decrease in thymidine incorporation provoked by shifting the cells in D-glucose-deprived medium were not dependent on the cell

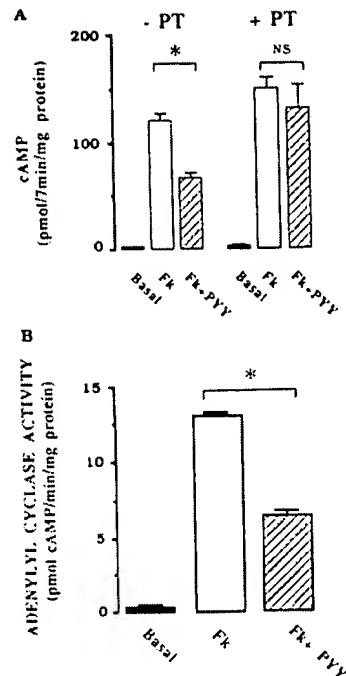


FIG. 4. Inhibition of forskolin-stimulated cAMP production and adenylate cyclase activity in PKSV-PCT cell membranes. PYY effect on cAMP production and adenylate cyclase activity was investigated on PKSV-PCT cells grown in D-glucose-enriched medium. A, cellular cAMP content from PKSV-PCT was determined on cells pretreated or not with 0.4 μ g/ml of pertussis toxin (\pm PT) for 18 h. Thereafter, cells were incubated in the absence of any compound (Basal), in the presence of 10^{-5} M forskolin (Fk), or with 10^{-5} M forskolin and 1 μ M PYY (Fk + PYY) for 7 min at 37 °C. The cAMP content was determined as described under "Experimental Procedures." Each value is the mean \pm S.E. of five determinations. B, membranes from PKSV-PCT cells were incubated in the absence of any compound (Basal), with 10^{-5} M forskolin (Fk), or with 10^{-5} M forskolin and 1 μ M PYY (Fk + PYY). After a 15-min incubation period, the reaction was stopped and adenylate cyclase activity measured as indicated under "Experimental Procedures." Each point is the mean \pm S.E. of five to seven determinations from three experiments. * $p < 0.001$ versus Fk and NS, nonsignificant.

density. This is illustrated in Fig. 6A for cell growth kinetics. These results led us to analyze the expression of PYY receptors under these three conditions of culture.

A dramatic decrease of [125 I]PYY binding to PKSV-PCT cell membranes was observed after substitution of D-glucose for oxaloacetate and pyruvate. Reintroduction of D-glucose in the culture medium completely restored the initial level of specific [125 I]PYY binding (Fig. 6C). The modulation of PYY receptor expression was not related to changes in the total protein content of PKSV-PCT cells which remained unchanged whatever the culture medium (data not shown). Nor was there any significant modification of basal adenylate cyclase activity (Table I) and of expression of the G protein subunits α_s , α_{i1} , α_{i2} , α_{i3} , and β , as assessed by Western blotting (Fig. 5). As a result of PYY receptor disappearance in quiescent cells cultured in D-glucose-deprived medium, PYY is no more able to inhibit forskolin-stimulated cAMP production (Table I). These results supported the idea that arrest of cell proliferation was associated with an almost total quenching of PYY receptor without any alteration of other components of the PYY-sensitive adenylate cyclase transmembrane signaling pathway.

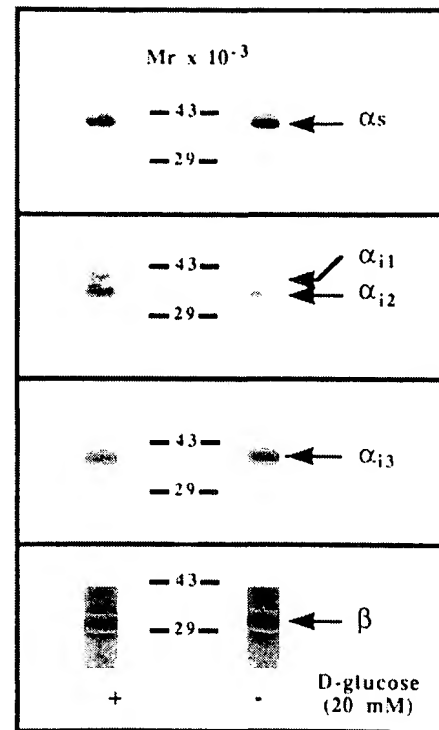


FIG. 5. Western blot analysis of α - and β -subunits of G_s and G_i proteins in membranes prepared from PKSV-PCT cells cultured in the presence or in the absence of D-glucose in the culture medium. Cell membranes were prepared from PKSV-PCT cells grown in D-glucose-enriched medium (+ D-glucose) or cultivated for 48 h in D-glucose-deprived medium (− D-glucose). Cell membrane proteins (50 μ g/lane) were subjected to 10% acrylamide slab gel. After transfer on nitrocellulose sheets, bands were revealed using antisera against the α_s , α_{i1}/α_{i2} , α_{i3} , and β - subunits of G proteins. For details see "Experimental Procedures."

Effect of PYY on Cell Growth and Incorporation of [3 H] Thymidine into DNA

The fact that PYY receptors were fully expressed on PKSV-PCT cells grown in the presence of D-glucose-enriched medium exhibiting high rates of cell doublings whereas they were no more expressed in quiescent state condition, raised the question of whether the peptide could itself interfere with cell proliferation in this particular model of cultured epithelial renal cells. To test this hypothesis, sets of PKSV-PCT cells were seeded on 35-mm plastic Petri dishes, and 2 days after seeding 10^{-7} M PYY was added to the D-glucose-enriched culture medium. Fig. 7A illustrates the cell growth kinetics in the presence or absence of PYY. PYY induced a slight increase in rates of cell growth which was maximal 4 days after addition of the peptide in the medium. Although discrete, the increase in cell number was constantly found. Results from three separate experiments showed that the maximal increase in cell number occurred after 3 days and ranged between 17 and 26%. In accordance with these results, the [3 H] thymidine incorporated in trichloroacetic acid-insoluble cell fractions was enhanced by $64 \pm 17\%$ ($n = 4$) after 24 h of exposure to PYY (Fig. 7B). The increase in [3 H] thymidine incorporation was also dependent on PYY concentration used. As shown in Fig. 7B, the half-maximal increase was obtained for 5 nM PYY with maximal increase for 10^{-7} M PYY.

Additional experiments were performed to test if pertussis toxin could blunt the observed increase in DNA synthesis

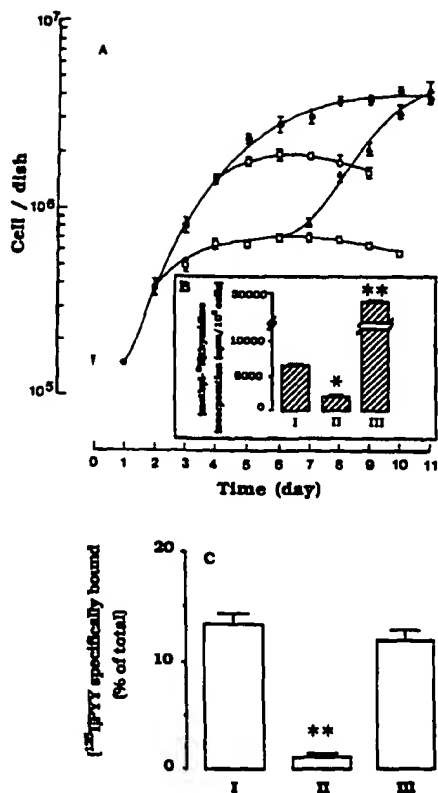


FIG. 6. Influence of D-glucose in the culture medium of PKSV-PCT cells on cell growth and expression of PYY receptor. A and B, cell growth kinetics of PKSV-PCT cells cultured in D-glucose-supplemented medium (●, condition I) exhibited a high rate of cell doubling. Transfer of the cells in D-glucose-deprived medium 2 or 4 days (○, condition II) after seeding (arrowhead) resulted in an arrest of cell growth. The phenomenon was reversed when glucose was reintroduced in the medium (▲, condition III). B, [methyl-³H]thymidine incorporation into DNA of PKSV-PCT cells in the three conditions described above (conditions I, II and III) was investigated. Values are the means \pm S.E. from triplicate counts. C, specific binding of [¹²⁵I]PYY to PKSV-PCT cell membranes prepared from cells cultured in the same conditions as described above: on D-glucose-enriched medium (I), in D-glucose-deprived medium (II), and after reintroduction of D-glucose in the D-glucose-deprived medium for 24 h (III). B and C, bars are the means \pm S.E. from three determinations performed in duplicate. * p < 0.005 versus basal condition I, and ** p < 0.001 versus condition I.

induced by PYY. For this purpose, PKSV-PCT cells grown for 2 days in D-glucose-enriched medium were incubated for 24 h in the presence or absence of 10^{-7} M PYY and/or 0.4 μ g/ml pertussis toxin (PT). In the absence of PYY, pertussis toxin reduced by 35% the [methyl-³H]thymidine incorporation (basal = $17,847 \pm 1,763$; +PT = $11,691 \pm 1,587$ cpm/ 10^6 cells, $n = 6$, $p < 0.005$). In the presence of PYY, [methyl-³H]thymidine incorporation increased by 45% the above described basal value, and addition of pertussis toxin significantly blunted by 32% the [methyl-³H]thymidine incorporated to a level similar to that measured in the absence of any agents (+PYY = $22,988 \pm 2,509$; +PYY and PT = $15,562 \pm 1,235$ cpm/ 10^6 cells, $n = 6$, $p < 0.001$). In this context, it is also worth pointing out that, in the presence of pertussis toxin, PYY did not significantly stimulate [methyl-³H]thymidine incorporation (+PT = $11,691 \pm 1,587$; +PYY and PT = $15,562 \pm 1,235$, $n = 6$, $p = 0.135$). Thus, these results strongly suggested that the activation of DNA synthesis induced by PYY was mediated by a pertussis toxin-sensitive G protein.

TABLE I

Effect of PYY on basal and forskolin-stimulated cAMP production in PKSV-PCT cells grown in D-glucose-enriched or D-glucose-deprived medium

Cellular cAMP production was measured in PKSV-PCT cells initially grown in D-glucose-enriched medium for 5 days or on same sets of cells cultured in D-glucose-deprived medium for 48 h as described in the legend of Fig. 4. Cells were incubated without (Basal) or with PYY (10^{-7} M), forskolin (Fk) (10^{-6} M) or PYY (10^{-7} M) and forskolin (10^{-6} M) for 7 min as described in "Experimental Procedures." Values are the means \pm S.E. of (n) determinations from three experiments.

Assay	cAMP	
	D-Glucose-enriched medium	D-Glucose-deprived medium
	pmol/7 min/mg protein	
Basal (4)	21.9 ± 2.1	15.3 ± 10.4
Fk (10^{-6} M) (6)	1204.5 ± 73.9^a	1199.4 ± 71.8^a
PYY (10^{-7} M) (5)	16.6 ± 1.8	13.6 ± 1.1
PYY (10^{-7} M) + Fk (10^{-6} M) (6)	667.4 ± 47.3^{ab}	1161.9 ± 100.3^a

^a p < 0.001 versus basal values.

^b p < 0.001 versus forskolin values.

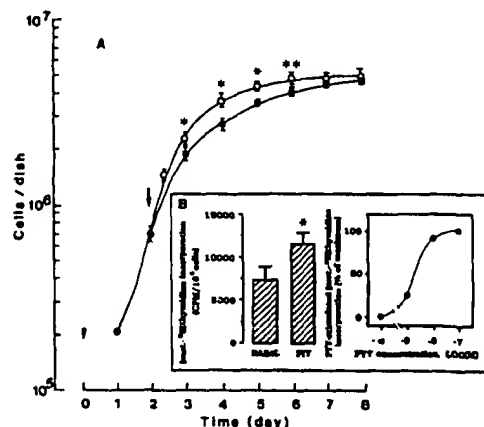


FIG. 7. Effect of PYY on PKSV-PCT cell growth and [methyl-³H]thymidine incorporation into DNA. A, representative growth kinetics from PKSV-PCT cells grown in the presence (○) or in the absence (●) of 10^{-7} M of PYY. Two days after seeding (arrowhead), PYY (arrow) was added to the D-glucose-enriched medium. Points are the means \pm S.E. from triplicate counts. B, [methyl-³H]thymidine incorporation into DNA of PKSV-PCT cells grown in D-glucose-enriched medium: effect of 10^{-7} M PYY. Left, 2 days after seeding cells were cultured in D-glucose-enriched medium containing or not 10^{-7} M PYY for 18 h. Cells were pulsed for the last 6 h of incubation with 0.5 μ Ci/ml [methyl-³H]thymidine as described under "Experimental Procedures." Values are the means \pm S.E. from triplicate counts from four experiments. Right, dose-effect of PYY (10^{-9} to 10^{-7} M) on [methyl-³H]thymidine incorporation into DNA of PKSV-PCT cells grown in D-glucose-enriched medium. Cells were incubated in the absence or in the presence of various concentrations of PYY in similar conditions described above. Points are the means of triplicate determinations from a single experiment. A and B, * p < 0.01 and ** p < 0.05.

DISCUSSION

The present paper demonstrates that PYY receptors are expressed in a growth-related manner in the renal PKSV-PCT cell line (17, 18) established from the early microdissected proximal tubules of L-PK/Tag1 transgenic mouse.

The PYY receptor of PKSV-PCT cells is a 46-kDa protein (see Fig. 3) that discriminates between PYY and NPY with a clear PYY-prefering profile (see Fig. 1A). It shares these molecular and pharmacological properties with the PYY

receptor previously characterized in rat small intestinal epithelium (1, 2, 7, 8). Further pharmacological identity between PYY receptors in PKSV-PCT cells (see Fig. 1A) and rat intestine (2) is indicated by their ability to bind the COOH-terminal (PYY(13-36)) fragment of PYY with a high affinity. This latter property is also displayed by the Y2-subtype of NPY receptors but not by the Y1-subtype (3, 4). In this connection, it should be noticed that a Y2-type of receptors with identical affinity for NPY and PYY has been previously identified in proximal tubule cells from the rabbit kidney (15, 29, 30). This Y2-receptor type is different from the PYY-preferring receptor described here in PKSV-PCT cells in that it has an identical affinity for NPY and PYY (29). However, these receptors display the same M_r , e.g. 46,000 (this paper and Ref. 30).

Direct evidence for the control of cAMP production by PYY action in PKSV-PCT cells is provided by the inhibitory effect of the peptide on forskolin-stimulated adenylylcyclase activity in membranes (see Fig. 4, A and B). Both inhibitory effects on cellular cAMP and membrane adenylylcyclase are reversed when cells have been pretreated with pertussis toxin, assessing for a classical pertussis toxin-sensitive pathway of G_i -mediated inhibition of adenylylcyclase. G_{i2} has been recently demonstrated to be responsible for coupling inhibitory receptors with adenylylcyclase in neuroblastoma \times glioma hybrid NG 108-5 cells (31, 32) and human platelets (31, 33). G_{i1} , G_{i2} , and G_{i3} are expressed in PKSV-PCT cells as shown by Western blot data (see Fig. 5). Results from this study do not allow to draw definitive conclusion on which subtype(s) of G_i is involved in PYY receptor-mediated response. In any case, our data clearly show that the PYY receptor in PKSV-PCT cells is directly coupled with a G_i protein. This is supported by the fact that GTP and pertussis toxin have the ability to modulate the binding of PYY to its receptors (see Fig. 1B). These experiments represent the first demonstration of a direct coupling of PYY receptors with a G protein since this was only speculated previously based on the ability of PYY to inhibit cAMP production in rat small intestinal epithelial crypt (8) and dog adipocyte (34).

Previous studies have shown that during epithelial cell migration from crypts to villi in rat small intestine, PYY receptors are present in proliferative crypt cells and thereafter the expression of PYY receptors is quenched resulting in the absence of receptors in quiescent differentiated enterocytes (8). In PKSV-PCT cells, the expression of Tag transcripts and thereby cell growth is placed under the control of a D-glucose-sensitive promoter and can be modulated by the presence or the absence of D-glucose in the culture medium without altering the tissue-specific functions of cells (17, 18). This cell line expressing PYY receptors (see above), is therefore most suitable to unravel the relationship between the process of epithelial cell proliferation and PYY receptors. We have shown that cell proliferation that follows transgene activation by D-glucose is associated with the expression of PYY receptors. When cells are cultured in the absence of D-glucose, large T antigen is no more expressed, cells stop to divide (17), and the expression of PYY receptors is dramatically reduced. This appears to be very specific for PYY receptors since total protein content of cells as well as functional and morphological properties of PKSV-PCT cells are unchanged, e.g. apical localization of aminopeptidase N, number and abundance of apical microvilli, villin content, and transepithelial resistance of filter-grown cells (17, 18). Nor was there any change in the expression of G_s and G_i protein subunits (see Fig. 5) or parathormone-stimulated cAMP production (17) showing that proteins involved in the cAMP

transmembrane signaling pathway are not altered when PKSV-PCT cells stop to divide. As a result of PYY receptor disappearance in quiescent cells, PYY is not able to inhibit cAMP production despite conservation of the G protein subunits and maintained adenylylcyclase response to forskolin (Table I). When glucose is reintroduced in the culture medium, PKSV-PCT cells start again to divide and PYY receptor binding activity is restored (see Fig. 6). This shows that the expression of PYY receptors is closely associated with the proliferative state of PKSV-PCT cells and is quite reversible in quiescent cells upon transgene activation. SV40 T antigen is known to regulate some cellular transcripts in a positive fashion (35). The mechanism whereby activation of T antigen results in concomitant cell growth and PYY receptor expression is not understood at the present time.

The exclusive expression of PYY receptors in proliferative PKSV-PCT cells as well as in intestinal crypt cells (8) raises the question of whether PYY has any role in the control of epithelial cell growth. Again, the PKSV-PCT cell line is a privileged model to address this issue. We have shown here that PYY promotes [*methyl*- 3H]thymidine incorporation into DNA and cell growth. The effect on DNA synthesis is dependent on PYY concentration and is abolished when cells have been pretreated with pertussis toxin (see Fig. 7C). This observation represents the first compelling evidence for a direct action of PYY on epithelial cell growth. Indeed such a growth-promoting effect of PYY had been only speculated (1) on the basis of correlation between plasma PYY levels and process of intestinal epithelial cell production in dietary manipulation studies, after partial small intestinal resection and after intravenous administration of epidermal growth factor (36-39). In this context, it is worth mentioning that a mitogenic effect of NPY was recently reported in rat vascular smooth muscle cells (40). The pathway that conveys PYY signal to the nuclei, where induction of genes needed for cell cycle progression and replication are executed, is not known. What is known is that pertussis toxin reverses PYY-induced DNA synthesis as well as PYY-induced inhibition of adenylylcyclase and of cAMP production (see Fig. 4, A and B). This observation suggests that pertussis toxin-sensitive G proteins are key mediators in the mitogenic action of PYY in PKSV-PCT cells but does not prove a role of cAMP in this action. Pertussis-toxin sensitive G proteins have been shown previously to be crucial for the mitogenic action of serotonin (41) and thrombin (42) in fibroblasts.

In conclusion, our studies indicated that a M_r 46,000 PYY receptor is expressed in a strict growth related manner in the proximal tubule PKSV-PCT cells when they proliferate upon transgene activation. Conversely, PYY promotes cell growth through a pertussis toxin-sensitive pathway. These findings reveal a new aspect of PYY action as a possible mediator in epithelial cell growth.

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